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Interleukin-6 receptor antagonists inhibit interleukin-11 biological activity

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ABSTRACT. The IL-6 receptor system comprises two functionally different chains: a binding chain (IL-6R) and a signal-transducing chain (gp130). The IL-6/IL-6R complexes associate with gp130, induce its dimerization and signal transduction. When IL-6 is complexed to IL-6R, two distinct sites of IL-6 are able to bind gp130. Other cytokines - oncostatin M (OM), leukemia inhibitory factor (LIF) or ciliary neurotrophic factor (CNTF) also use the gp130 transducer and induce its heterodimerization with LIF receptor (LIFR).
A series of IL-6 mutants have been generated which function as IL-6 receptor antagonists (IL-6RA). These IL-6RA carried substitutions that increased their affinity with IL-6R and abolished 1 or the 2 sites of interaction with gp130. All the IL-6RA inhibited wild-type IL-6. The IL-6RA with one mutated binding site to gp130 inhibited IL-11 activity. They did not affect those of CNTF, LIF and OM, even when used at a very high concentration at which virtually all membrane IL-6R were bound to IL-6RA. IL-6RA with two mutated gp130 binding sites did not affect IL-11, CNTF, LIF or OM activities. The results indicate that the interaction of one gp130 chain with IL-6R/IL-6R complexes inhibited further the dimerization of gp130 induced by IL-11/IL-11R but not its heterodimerization with LIFR. Thus these IL-6RA can also function as IL-11 antagonists.

Key words: Interleukin-6, interleukin-11, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, multiple myeloma

INTRODUCTION

The family of cytokines sharing a common transducer chain with interleukin (IL)-6 comprises five members: cardiotropin-1 (CT-I), ciliary neurotrophic factor (CNTF), IL-11, leukemia inhibitory factor (LIF) and oncostatin M (OM) [1]. These cytokines exert a large spectrum of identical biological activities through their common activation of the gp130 transducer which is expressed on almost every cell *in vivo* [1]. gp130 transducer is necessary for the development of embryos since inactivation of gp130 gene yields to early embryonic death due to failure of cardiac muscle development [2]. The most studied member of this family is IL-6. Owing to its large spectrum of activity on normal cells, this cytokine is involved in numerous pathological processes [3 - 5]. In particular, IL-6 has been shown to promote the growth of several cancer cells: HIV associated lymphoma [6], multiple myeloma [7] and renal cancer [8]. Overproduction of IL-6 is also responsible for the inflammatory and cachectic syndromes associated with tumor development [9]. Finally, IL-6 is involved in post-menauposis osteoporosis [10]. For these reasons various antagonists to IL-6 have been developed: mutated IL-6 [11], antibodies to IL-6 [12] or IL-6R [13]. The development of IL-6 receptor antagonists (IL-6RA) is based on the binding property of IL-6 to the IL-6R and the gp130 transducer. IL-6 first binds to IL-6R with an affinity of 10^{-9} M [14] through a binding site previously-called site 1 [15]. The IL-6/IL-6R complex plays no role in signalling. It binds to the gp130 transducer resulting in the formation of a hexameric complex comprising two gp130 transducer chains, two IL-6R and two IL-6 proteins [16, 17]. In this hexameric complex, IL-6 binds to each gp130 chain through two different sites called site 2 and site 3 [11]. Site 2 is formed by exposed residues in the IL-6 A and C helices and corresponds to a receptor binding site conserved in several cytokines, including growth hormone [15, 18, 19]. Site 3 is a composite binding site formed by residues at the beginning of D helix spatially flanked by residues in the initial part of A-B loop [20, 21]. Amino acid substitutions at either of these two sites do not substantially effect binding to IL-6R but decreases interaction of the IL-6/IL-6R complex with one of the two gp130 chains.

Several IL-6RA have been generated by mutating site 2 or site 2+3 resulting in different binding to gp130 [22]. In addition, the binding site to IL-6R was also mutated in order to increase the affinity with IL-6R [15]. These IL-6RA proved to be efficient inhibitors

of IL-6 activity on various cells [22]. In this report, we show that IL-6RA with mutated site 2 - which were able to prevent gp130 dimerization - also inhibited IL-11 activity without affecting CNTF, LIF or OSM activities. On the contrary, IL-6RA with mutated sites 2+3 - that failed to bind gp130-inhibited IL-6 but did not affect IL-11 biological activity.

MATERIALS AND METHODS

Production of IL-6 antagonists. The characteristics of the IL-6RA have been described in detail [11, 22] and are summarized in the Table. Briefly, DFRD has four mutations in one binding site to gp130 (site 2) at positions 31, 35, 118 and 121. Sant1 and Sant5 antagonists have the same mutated site 2 and additional mutations in the binding site to IL-6R (site 1) that increased their affinity with IL-6R by 4.5 and 40 fold respectively. DFRD, Sant1 and Sant5 when complexed to IL-6R are still able to bind one gp130 transducer but could not induce gp130 dimerization due to the mutated site 2. Sant7 has the same mutations as Sant 5 and additional mutations in the second binding site to gp130 (site 3). Thus the complex Sant7/IL-6R is unable to bind to any gp130 transducer. Sant8 has the same mutations as Sant5 and additional mutations in site 1 inducing a stronger affinity with IL-6R. Sant9 has the mutated sites of DFRD and two additional mutations in site 1.

Cell lines. The XG-4CNTF and XG-6 myeloma cell lines were obtained in our laboratory [23, 24]. The growth of XG-4CNTF is dependent upon addition of one of the gp130-activating cytokines: CNTF, IL-6, IL-11, LIF or OM. The growth of XG-6 is supported by IL-6, IL-11, LIF and OM. These cell lines were free of mycoplasma contamination.

Biological assay. XG-4-CNTF and XG-6 cells were washed twice with RPMI1640 culture medium, incubated for 5 hrs at 37°C in RPMI1640, washed again twice, and cultured for 5 days in 96 well flat-bottomed microplates in RPMI1640 medium supplemented with 10% of fetal calf serum in the presence of cytokine concentration inducing a maximal proliferation. The concentrations of the cytokines used with XG-4CNTF cells were: 30 pg/ml of IL-6, 30 ng/ml of IL-11, 100 pg/ml of CNTF, 100 pg/ml of LIF or 100 pg/ml of OM. Those used with XG-6 cells were: 100 pg/ml of IL-6, 10 ng/ml of IL-11, LIF or OM. Graded concentrations of IL-6RA were added at the beginning of the cultures. 0.5 µCi of tritiated thymidine (25 Ci/mM, CEA, Saclay, France) were added for the last 8 hrs of cultures, and tritiated thymidine incorporation was determined.

Reagents. Purified IL-6 was provided by D. Stinchcomb (Synergen, Boulder, CO, USA), OM by M. Shoyab (Bristol Myers Squibb, Seattle, WA, USA) and CNTF by G. Yancopoulos (Regeneron, Tarrytown, NY, USA). LIF and IL-11 were purchased from R&D (Minneapolis, MN, USA).

RESULTS AND DISCUSSION

The proliferation of XG-4CNTF or XG-6 cells in the presence of graded concentrations of the different gp130 activating cytokines is outlined in Figs. 1A and 1B. As previously published, the proliferation of XG-4CNTF cells is supported by the 5 gp130-activating cytokines: CNTF, IL-6, IL-11, LIF and OSM (Fig. 1A). XG-4CNTF cells expressed the CNTF receptor unlike XG-6 cells [23]. The proliferation of XG-6 cells was supported by IL-6, IL-11, LIF and OM (Fig. 1B). In previous experiments, we have shown that the growth activity of CNTF, OSM, LIF and IL-11 was unaffected by anti-IL-6R or anti-IL-6 antibodies but completely inhibited by anti-gp130 antibodies [23, 25].

The effects of the different IL-6RA on the biological activity of the gp130-activating cytokines are shown in Fig. 2 and Fig. 3. The IL-6RA with mutated site 2 (DFRD, Sant1, Sant5, Sant8, Sant9) or site 2 + 3 (Sant7) strongly inhibited IL-6 activity. For the XG-4CNTF human myeloma cell line, the DFRD antagonist was as potent as the other antagonists despite its lower affinity with IL-6R (Table 1). For the XG-6 human myeloma cell line, it was less potent in agreement with our previous results [22]. We have no clear explanation for this result. It is noteworthy that the XG-4CNTF human myeloma cell line is highly sensitive to IL-6 [23] eventually due to some mutations in IL-6R resulting in stronger affinity with IL-6 or IL-6RA. Sant7 with mutated sites 2 + 3 was less potent than Sant5 and Sant8 that have a similar affinity with IL-6R (Table 1). The results are in agreement with our previous findings [22]. This is understantable since the

Table 1

Substitutions																				
	Antagonists				Superbinder														slL-6Ra	sgp130	sgp130
Mutant protein	Y 31	G 35	S 118	V 121	L 57	E 59	N 60	K 66	A 68	E 69	K 70	Q 75	S 76	Q 175	S 176	Q 183	binding activity *	binding activity §	dimerizing activity §
DFRD	D	F	R	D													1+0.1	+	-
Sant I	D	F	R	D										I	R	A	4.5+0.6	+	-
Sant 5	D	F	R	D								Y	K	I	R	A	40+3.4	+	-
Sant 7	D	F	R	D	D	F	W					Y	K	I	R	A	65+7	-	-
Sant 8	D	F	R	D				V	R	M	E	Y	K	I	R	A	70+8	+	-
Sant 9	D	F	R	D								Y	K				12+1.1	+	-

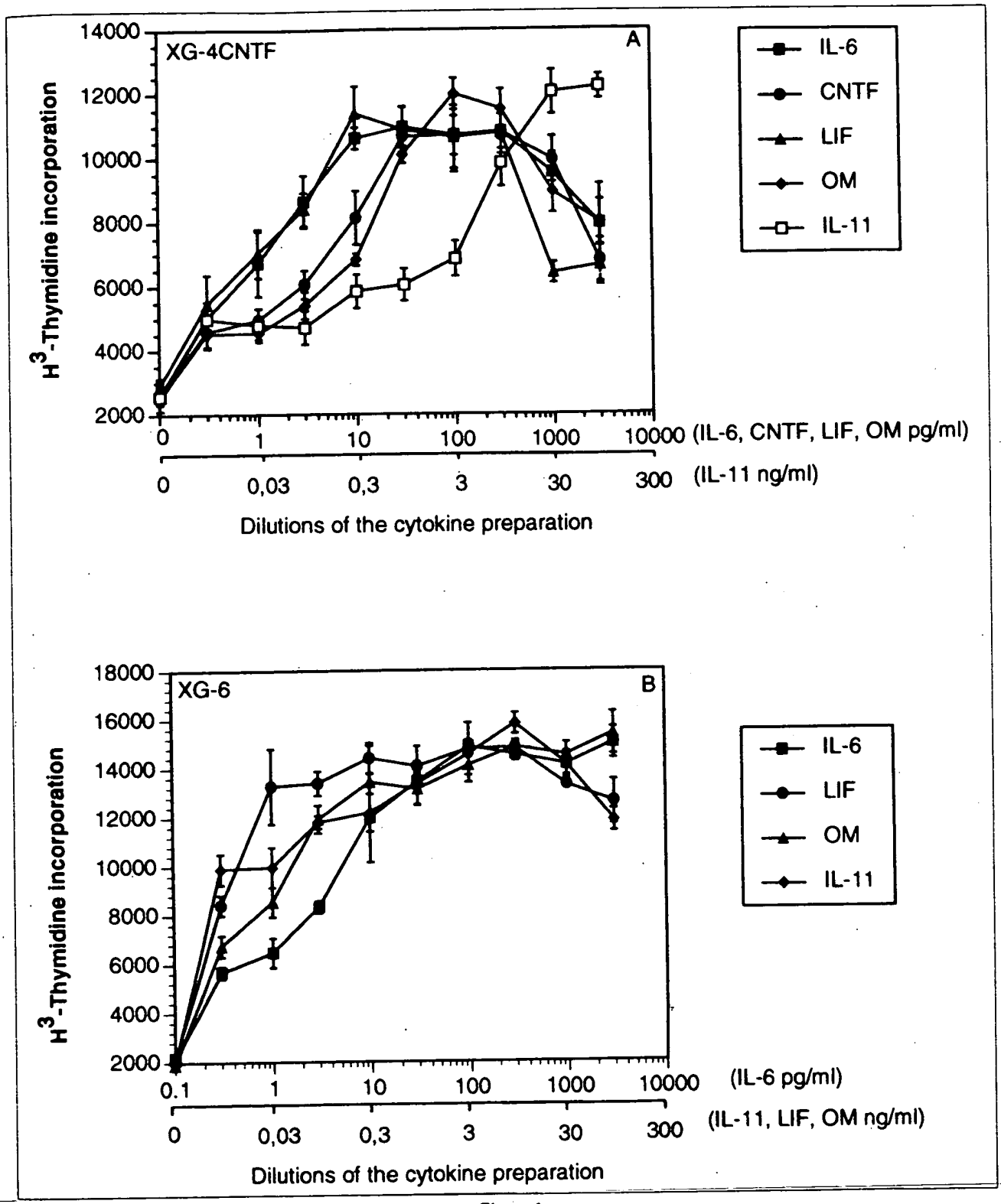

Figure 1

Proliferation response of XG-4-CNTF and XG-6 cells to the various gp130 cytokines

Ten thousand XG-4CNTF (A) or XG-6 (B) cells were cultured for 5 days in 100 µl culture medium in the presence of various cytokine concentrations. Results are the mean of tritiated thymidine incorporations determined after 5 days of culture on six separate culture wells.

binding of Sant5/IL-6R complexes to one gp130 transducer probably stabilizes these complexes compared to Sant7/IL-6R complexes unable to bind gp130.

None of these antagonists inhibited CNTF, LIF or OM activies. These three cytokines use the gp130 transducer in association with another receptor, the LIFR, and eventually with another uncharacterized

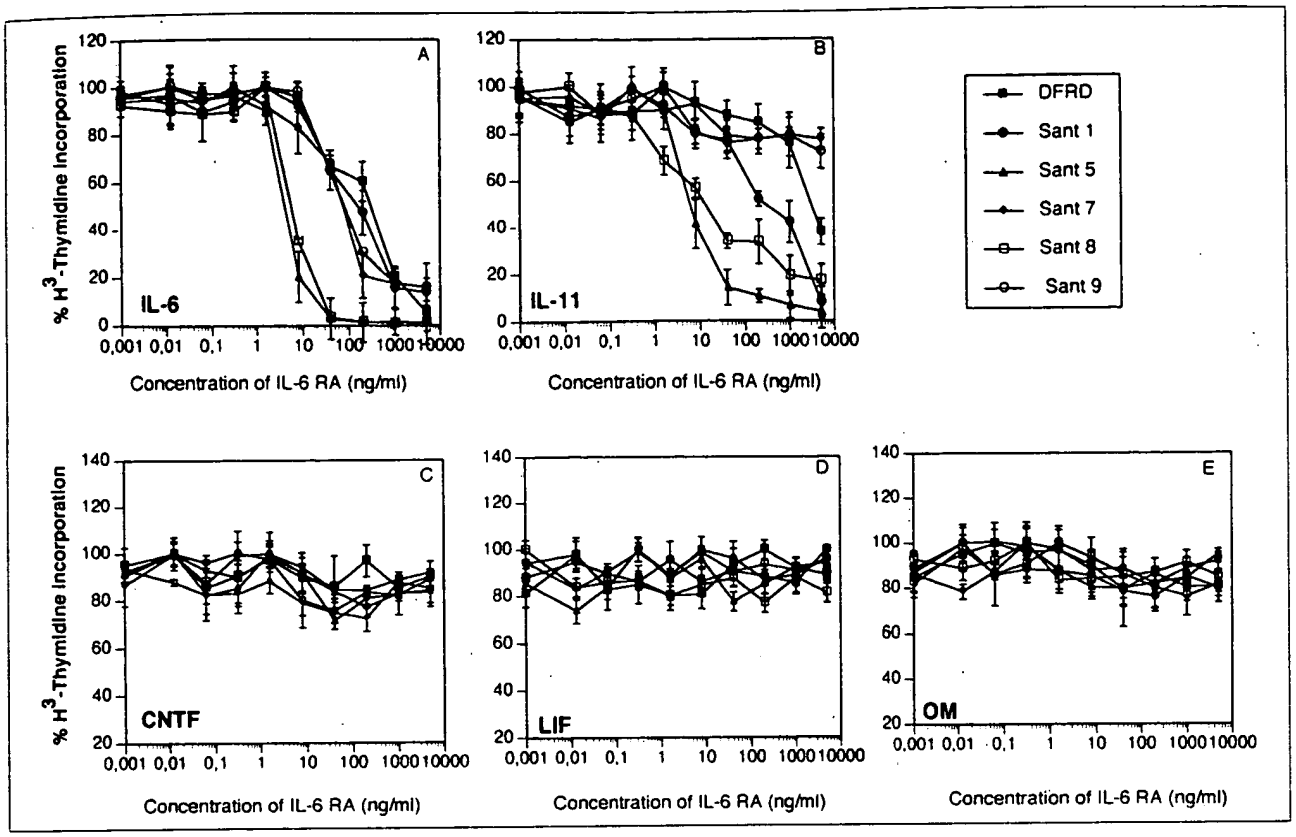

Figure 2

Inhibition of XG-4CNTF proliferation by the IL-6 receptor antagonists (IL-6RA)

Ten thousand XG-4-CNTF cells were cultured in 100 µl of culture medium for 5 days with either 30 pg/ml of IL-6 (A), or 30 ng/ml of IL-11 (B), or 100 pg/ml of CNTF (C), or 100 pg/ml of LIF (D), or 100 pg/ml of OM (E). Graded concentrations of the various IL-6RA were added at the beginning of the culture. Results are the mean, of tritiated thymidine incorporations determined at the end of the culture on six separate culture cells.

receptor for OM [1, 26]. The lack of inhibitory activity of these IL-6RA was observed at very high concentrations (up to 30 µg/ml). At these concentrations (i.e. 10000 fold the Kd of IL-6RA with IL-6), virtually all membrane IL-6R were occupied by mutated IL-6. In the case of IL6RA with site 2 mutation (DFRD, Sant1, Sant5, Sant8), the complex IL-6RA/IL-6R may bind to one gp130 transducer and this binding does not prevent the heterodimerization of gp130 and LIFR induced by CNTF, LIF or OM. Interestingly, these site 2 antagonists with one remaining binding site to gp130 are able to completely block IL-11 activity whereas the Sant7 IL-6RA with mutated sites 2 and 3 (not able to bind gp130) has no inhibitory activity. How to interpret these results by taking into account the recent knowledge of the biology of IL-11R whose gene was recently cloned [27]. Similar to IL-6R, the soluble form of IL-11R is able to bind IL-11 and to confer a functional activity to IL-11 [27]. A recent study has indicated that the IL-11/IL-11R complex directly binds to gp130 through at least two sites [28]. However, unlike IL-6/IL-6R complexes, the IL-11/IL-11R complex is unable to trigger gp130 dimerization and might involve another transducer chain that is not LIFR [28]. Our results indicate that the occupancy of one binding site to gp130 by the IL-6RA/IL-6R complexes prevents the binding of IL-11/IL-11R complex to gp130. Dr Brochier's group (personal communication)has recently obtained monoclonal antibodies to gp130 recognizing 2 different epitopes of the gp130 involved in the binding of IL-6/IL-6R complex to gp130 [29]. It would be interesting to examine whether one of these epitopes is involved in the binding of IL-11/IL-11R complex to gp130.

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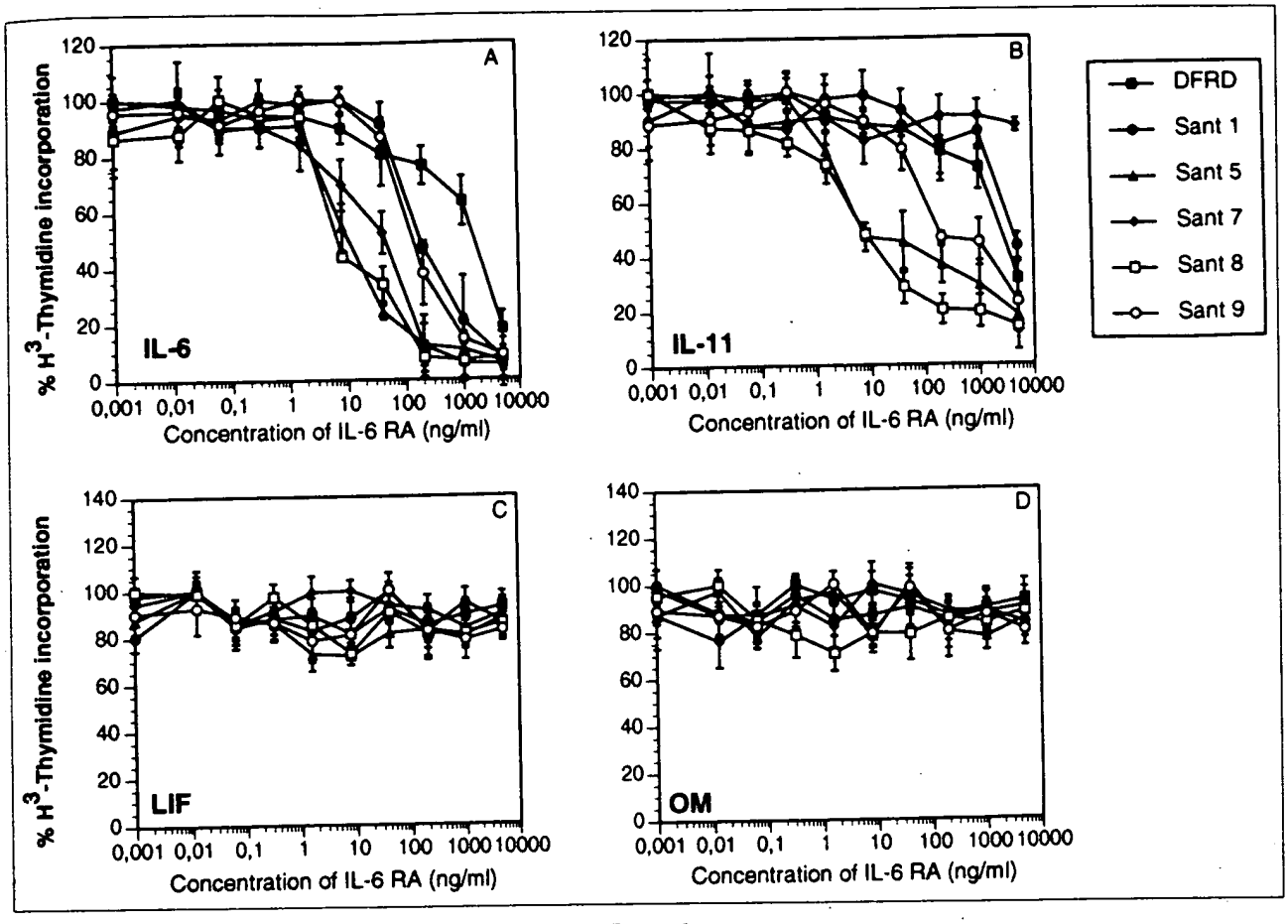

Figure 3
Inhibition of XG-6 proliferation by IL-6 receptor antagonists (IL-6RA)
Ten thousand XG-6 cells were cultured in 100 µl of culture medium for 5 days with either 100 pg/ml of IL-6 (A), or 10 ng/ml of IL-11 (B), LIF (C) or OM (D). Graded concentrations of the various IL-6RA were added at the beginning of the culture. Results are the mean, of tritiated thymidine incorporations determined at the end of the culture on six separate culture wells.

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